9. MUTAGENICITY

Since 1978, more than 100 publications have appeared in which genotoxicity assays were used with diesel emissions, the volatile and particulate fractions (including extracts), or individual chemicals found in diesel emissions. Although most of the studies deal with the question of whether particulate extracts from diesel emissions possess mutagenic activity in microbial and mammalian cell assays, a number of studies in recent years have employed bioassays (most commonly *Salmonella* TA98 without S9) to evaluate (1) extraction procedures, (2) fuel modifications, (3) bioavailability of chemicals from diesel particulate matter (DPM), and (4) exhaust filters or other modifications and other variables associated with diesel emissions. This chapter will focus on the application of the available data to issues of genetic risk assessment; reports dealing with mutagenic activity associated with the metabolism of particular chemicals of DPM are discussed in Chapter 10. Also, because of the large number of reports, this discussion will focus on key references. An International Agency for Research on Cancer (IARC) monograph (International Agency for Research on Cancer, 1989) contains an exhaustive description of the available studies and other review articles (Claxton, 1983; Pepelko and Peirano, 1983); the proceedings of several symposia on the health effects of diesel emissions (U.S. EPA, 1980; Lewtas, 1982; Ishinishi et al., 1986; International Agency for Research on Cancer, 1989) are also available.

9.1. GENE MUTATIONS

Huisingh et al. (1978) demonstrated that dichloromethane extracts from DPM were mutagenic in strains TA1537, TA1538, TA98, and TA100 of *S. typhimurium*, both with and without rat liver S9 activation. This report contained data from several fractions as well as DPM from different vehicles and fuels. Similar results with diesel extracts from various engines and fuels have been reported by a number of investigators using the *Salmonella* frameshift-sensitive strains TA1537, TA1538, and TA98 (Siak et al., 1981; Claxton, 1981; Dukovich et al., 1981; Brooks et al., 1984). Similarly, mutagenic activity was observed in *Salmonella* forward mutation assays measuring 8-azaguanine resistance (Claxton and Kohan, 1981) and in *E. coli* mutation assays (Lewtas, 1983).

One approach to identifying significant mutagens in chemically complex environmental samples such as diesel exhaust or ambient particulate extracts is the combination of short-term bioassays with chemical fractionation (Scheutzle and Lewtas, 1986). The analysis is most frequently carried out by sequential extraction with increasingly polar or binary solvents. Prefractionation by silica-column chromatography separates compounds by polarity or into acidic, basic, and neutral fractions. The resulting fractions are too complex to characterize by chemical

methods, but the bioassay analysis can be used to determine fractions for further analysis. In most applications of this concept, *Salmonella* strain TA98 without the addition of S9 has been used as the indicator for mutagenic activity. Generally, a variety of nitrated polynuclear aromatic compounds have been found that account for a substantial portion of the mutagenicity (Liberti et al., 1984; Schuetzle and Frazer, 1986; Schuetzle and Perez, 1983). However, not all bacterial mutagenicity has been identified in this way, and the identity of the remainder of the mutagenic compounds remains unknown. The nitrated aromatics thus far identified in diesel exhaust were the subject of review in the IARC monograph on diesel exhaust (International Agency for Research on Cancer, 1989). In addition to the simple qualitative identification of mutagenic chemicals, several investigators have used numerical data to express mutagenic activity as activity per distance driven or mass of fuel consumed. These types of calculations have been the basis for estimates that the nitroarenes (both mono- and dinitropyrenes) contribute a significant amount of the total mutagenic activity of the whole extract (Nishioka et al., 1982; Salmeen et al., 1982; Nakagawa et al., 1983). However, as noted by Claxton (1983), because most of these studies used only strain TA98 without exogenous activation, several classes of mutagenic chemicals may have gone undetected.

Matsushita et al. (1986) tested particle-free diesel exhaust gas and a number of benzene nitro-derivatives and polycyclic aromatic hydrocarbons (PAHs) (many of which have been identified as components of diesel exhaust gas). The particle-free exhaust gas was positive in both TA100 and TA98, but only without S9 activation. Of the 94 nitrobenzene derivatives tested, 61 were mutagenic, and the majority showed greatest activity in TA100 without S9. Twenty-eight of 50 PAHs tested were mutagenic, all required the addition of S9 for detection, and most appeared to show a stronger response in TA100. When 1,6-dinitropyrene was mixed with various PAHs or an extract of heavy-duty (HD) diesel exhaust, the mutagenic activity in TA98 was greatly reduced when S9 was absent but was increased significantly when S9 was present. These latter results suggested that caution should be used in estimating mutagenicity (or other toxic effects) of complex mixtures from the specific activity of individual components.

Mitchell et al. (1981) reported mutagenic activity of DPM extracts of diesel emissions in the mouse lymphoma L5178Y mutation assay. Positive results were seen both with and without S9 activation in extracts from several different vehicles, with mutagenic activity only slightly lower in the presence of S9. These findings have been confirmed in a number of other mammalian cell systems using several different genetic markers. Casto et al. (1981), Chescheir et al. (1981), Li and Royer (1982), and Brooks et al. (1984) all reported positive responses at the HGPRT locus in Chinese hamster ovary (CHO) cells. Morimoto et al. (1986) used the APRT and Oua^r loci in CHO cells; Curren et al. (1981) used Oua^r in BALB/c 3T3 cells. In all of these studies, mutagenic activity was observed without S9 activation. Liber et al. (1981) used the thymidine

kinase (TK) locus in the TK6 human lymphoblast cell line and observed induced mutagenesis only in the presence of rat liver S9 when testing a methylene chloride extract of diesel exhaust. Barfnecht et al. (1982) also used the TK6 assay to identify some of the chemicals responsible for this activation-dependent mutagenicity. They suggested that fluoranthene, 1-methylphenanthrene, and 9-methylphenanthrene could account for over 40% of the observed activity.

Morimoto et al. (1986) injected DPM extracts (250 to 4,000 mg/kg) into pregnant Syrian hamsters and measured mutations at the APRT locus in embryo cells cultivated 11 days after i.p. injection. Neutral fractions from both light-duty (LD) and HD tar samples resulted in increased mutation frequency at 2,000 and 4,000 mg/kg. Belisario et al. (1984) applied the Ames test to urine from Sprague-Dawley rats exposed to single applications of DPM administered by gastric intubation, i.p. injection, or s.c. gelatin capsules. In all cases, dose-related increases were seen in TA98 (without and with S9) from urine concentrates taken 24 h after particle administration. Urine from Swiss mice exposed by inhalation to filtered exhaust (particle concentration 6 to 7 mg/m^3) for 7 weeks (Pereira et al., 1981a) or Fischer 344 rats exposed to DPM (2 mg/m^3) for 3 months to 2 years was negative in *Salmonella* strains. Because of the large differences in dosages, these findings should not be construed as conflicting.

Schuler and Niemeier (1981) exposed *Drosophila* males in a stainless steel chamber connected to the 3 m^3 chamber used for the chronic animal studies at EPA (see Hinners et al., 1980 for details). Flies were exposed for 8 h and mated to untreated females 2 days later. Although the frequency of sex-linked recessive lethals from treated males was not different from that of controls, the limited sample size precluded detecting less than a threefold increase over controls. The authors noted that, because there were no signs of toxicity, the flies might tolerate exposures to higher concentrations for longer time periods.

Specific-locus mutations were not induced in (C3H × 101)F_1 male mice exposed to diesel exhaust 8 h/day, 7 days/week for either 5 or 10 weeks (Russell et al., 1980). The exhaust was a 1:18 dilution and the average particle concentration was 6 mg/m^3. After exposure, males were mated to T-stock females and matings continued for the reproductive life of the males. The results were unequivocally negative; no mutants were detected in 10,635 progeny derived from postspermatogonial cells or in 27,917 progeny derived from spermatogonial cells.

9.2. CHROMOSOME EFFECTS

Mitchell et al. (1981) and Brooks et al. (1984) reported increases in sister chromatid exchanges (SCE) in CHO cells exposed to DPM extracts of emissions from both LD and HD diesel engines. Morimoto et al. (1986) observed increased SCE from both LD and HD DPM extracts in PAH-stimulated human lymphocyte cultures. Tucker et al. (1986) exposed human peripheral lymphocyte cultures from four donors to direct diesel exhaust for up to 3 h. Exhaust

was cooled by pumping through a plastic tube about 20 feet long; airflow was 1.5 L/min. Samples were taken at 16, 48, and 160 min of exposure. Cell cycle delay was observed in all cultures; significantly increased SCE levels were reported for two of the four cultures. Structural chromosome aberrations were induced in CHO cells by DPM extracts from a Nissan diesel engine (Lewtas, 1983) but not by similar extracts from an Oldsmobile diesel engine (Brooks et al., 1984).

Pereira et al. (1981a) exposed female Swiss mice to diesel exhaust 8 h/day, 5 days/week for 1, 3, and 7 weeks. The incidence of micronuclei and structural aberrations was similar in bone marrow cells of both control and exposed mice. Increased incidences of micronuclei, but not SCE, were observed in bone marrow cells of male Chinese hamsters after 6 months of exposure to diesel exhaust (Pereira et al., 1981b).

Guerrero et al. (1981) observed a linear concentration-related increase in SCE in lung cells cultured after intratracheal instillation of DPM at doses up to 20 mg/hamster. However, they did not observe any increase in SCE after 3 months of inhalation exposure to diesel exhaust particles (6 mg/m^3).

Pereira et al. (1982) measured SCE in embryonic liver cells of Syrian hamsters. Pregnant females were exposed to diesel exhaust (containing about 12 mg/m^3 particles) from days 5 to 13 of gestation or injected intraperitoneally with diesel particles or particle extracts on gestational day 13 (18 h before sacrifice). Neither the incidence of SCE nor mitotic index was affected by exposure to diesel exhaust. The injection of DPM extracts but not DPM resulted in a dose-related increase in SCE; however, the toxicity of the DPM was about twofold greater than the DPM extract.

In the only studies with mammalian germ cells, Russell et al. (1980) reported no increase in either dominant lethals or heritable translocations in males of T-stock mice exposed by inhalation to diesel emissions. In the dominant lethal test, T-stock males were exposed for 7.5 weeks and immediately mated to females of different genetic backgrounds (T-stock; [C3H × 101]; [C3H × C57BL/6]; [SEC × C57BL/6]). There were no differences from controls in any of the parameters measured in this assay. For heritable translocation analysis, T-stock males were exposed for 4.5 weeks and mated to (SEC × C57BL/6) females, and the F_1 males were tested for the presence of heritable translocations. Although no translocations were detected among 358 progeny tested, the historical control incidence is less than 1/1,000.

9.3. OTHER GENOTOXIC EFFECTS

Pereira et al. (1981b) exposed male strain A mice to diesel exhaust emissions for 31 or 39 weeks using the same exposure regimen noted in the previous section. Analyses of caudal sperm for sperm-head abnormalities were conducted independently in three separate laboratories. Although the incidence of sperm abnormalities was not significantly above controls in any of the

three laboratories, there were extremely large differences in scoring among the three (control values were 9.2%, 14.9%, and 27.8% in the three laboratories). Conversely, male Chinese hamsters exposed for 6 mo (Pereira et al., 1981c) exhibited almost a threefold increase in sperm-head abnormalities. It is noted that the control incidence in the Chinese hamsters was less than 0.5%. Hence, it is not clear whether the differing responses reflect true species differences or experimental artifacts.

9.4. SUMMARY

Extensive studies with *Salmonella* have unequivocally demonstrated mutagenic activity in both particulate and gaseous fractions of diesel exhaust. In most of the studies using *Salmonella*, DPM extracts and individual nitropyrenes exhibited the strongest responses in strain TA98 when no exogenous activation was provided. Gaseous fractions reportedly showed greater response in TA100, whereas benzo[a]pyrene and other unsubstituted PAHs are mutagenic only in the presence of S9 fractions. The induction of gene mutations has been reported in several in vitro mammalian cell lines after exposure to extracts of DPM. Note that only the TK6 human cell line did not give a positive response to DPM extracts in the absence of S9 activation. Mutagenic activity was recovered in urine from animals treated with DPM by gastric intubation and i.p. and s.c. implants, but not by inhalation of DPM or diluted diesel exhaust. Dilutions of whole diesel exhaust did not induce sex-linked recessive lethals in *Drosophila* or specific-locus mutations in male mouse germ cells.

Structural chromosome aberrations and SCE in mammalian cells have been induced by particles and extracts. Whole exhaust induced micronuclei but not SCE or structural aberrations in bone marrow of male Chinese hamsters exposed to whole diesel emissions for 6 mo. In a shorter exposure (7 weeks), neither micronuclei nor structural aberrations were increased in bone marrow of female Swiss mice. Likewise, whole diesel exhaust did not induce dominant lethals or heritable translocations in male mice exposed for 7.5 and 4.5 weeks, respectively.

Mutagenicity data have been applied both to issues of heritable genetic risk and somatic cell effects, most notably cancer. For heritable genetic effects, the U.S. Environmental Protection Agency's Guidelines for Mutagenicity Risk Assessment (U.S. EPA, 1987) are applicable here. The mammalian germ-cell studies measuring defined genetic endpoints conducted on diesel emissions have shown negative results, but the sample size in the heritable translocation test is too small for a meaningful conclusion. In the absence of definitive mammalian germ-cell results, the guidelines recommend that mutagenic activity and the ability to interact with mammalian germ cells be evaluated separately. As stated, the presence of a large number of mutagenic chemicals in diesel emissions is unambiguous. Sperm abnormality assays are presumably the only other source of data on the interaction of diesel emissions with mammalian germ cells. The negative response

in the mouse is in apparent conflict with the positive observation in the hamster, and there is not sufficient information to resolve this discrepancy. Hence, the questions of germ-cell interaction and the potential for human germ-cell mutagenic risk of diesel emissions remain unanswered.

The application of genotoxicity information to the question of the potential carcinogenicity of chemical agents was initially based on the premise that somatic mutation is an integral step in the carcinogenic process. However, unlike the situation for germ-cell mutagenicity, assays are not weighted strictly by their biological relationship to the particular species, sex, or tissue site of cancer. The size of the database and the degree of correlation of genotoxicity test results with animal cancer bioassays are frequently given great weight. Indeed, a common conclusion of the efforts of the National Toxicology Program on the use of in vitro assays is that no single in vitro genotoxicity test or battery of tests (among the four assays in their program) improves on the performance of the *Salmonella* assay in predicting rodent carcinogenicity of an untested chemical. When rodent carcinogenicity data are available, phylogenetic and other biological aspects of the genotoxicity data are important considerations in the weight-of-evidence process. With diesel emissions, additional complications arise because of the chemical complexity of the material being tested. Although it is clear that several of the individual chemical constituents of diesel exhaust have been demonstrated to be both mutagenic and carcinogenic, it is likely that the constituents responsible for the mutational increases observed in bacteria are different from those responsible for the observed increases in CHO cells (Li and Dutcher, 1983) or in human hepatoma-derived cells (Eddy et al., 1986). Chapter 10 deals more thoroughly with metabolism and mechanisms of carcinogenesis.

9.5. REFERENCES

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